Biochemical Systematics and Ecology 48 (2013) 238–247

Contents lists available at SciVerse ScienceDirect

Biochemical Systematics and Ecology

journal homepage: www.elsevier.com/locate/biochemsyseco

Genetic diversity of *Brachystegia boehmii* Taub. and *Burkea africana* Hook. f. across a fire gradient in Niassa National Reserve, northern Mozambique

Ivete Maquia [a], Natasha S. Ribeiro [b], Vando Silva [a], Fernanda Bessa [c], Luis F. Goulao [c], Ana I. Ribeiro [c,*]

[a] *Biotechnology Center, Eduardo Mondlane University, Av. de Moçambique Km 1.5, Maputo, Mozambique*
[b] *Faculty of Agronomy and Forest Engineering, Eduardo Mondlane University, Av. J. Nyerere 3453/Campus Universitário Principal, Building #1, Maputo, Mozambique*
[c] *Tropical Research Institute/BioTrop, Quinta do Marquês, 2784-505 Oeiras, Portugal*

ARTICLE INFO

Article history:
Received 5 September 2012
Accepted 26 December 2012
Available online 7 February 2013

Keywords:
Brachystegia boehmii
Burkea africana
Genetic diversity
Inter simple sequence repeats (ISSR)
Miombo woodlands
Mozambique

ABSTRACT

Miombo woodlands constitute the most important type of vegetation in southern Africa, covering about 70% of the Zambezian phytoregion. This ecosystem, dominated by the genera *Brachystegia*, *Julbernardia* and *Isoberlinia*, has an immeasurable socio-economic and environmental value, playing a key role in formal and informal economies and in energy, water and carbon balances. Anthropogenic fires represent one of the major threats, compromising the stability of miombo. In this study we report on the usefulness of ISSR markers to assess, for the first time, the genetic diversity in two typical miombo species, *Brachystegia boehmii* Taub. and *Burkea africana* Hook. f. across a fire gradient in the Niassa National Reserve (NNR). According to our data, ISSR seem to be a suitable molecular marker's system for biodiversity studies in both species, generating high levels of polymorphisms coupled with a convenient resolving power. The results point to a link between fire-tolerance and genetic diversity, as judged by the higher diversity levels observed in *B. africana* (fire-tolerant) and by the evolutive fire response of *B. boehmii*. Although fire differentially affects the biodiversity in each species, in general, the overall genetic diversity was high and their survival does not seem to be compromised by the frequency of fires, agreeing with the fact that NNR is one of the least disturbed areas of deciduous miombo.

1. Introduction

Miombo woodlands are the most widespread forest type in southern Africa, covering approximately 2.4 million km^2 across seven countries: Angola, DR Congo, Malawi, Mozambique, Tanzania, Zambia and Zimbabwe. The resources of the woodlands are crucial to the livelihood systems of millions of rural and urban dwellers (Dewees et al., 2011). Besides its social importance, miombo contributes to formal economies, providing valuable sources of wood, and plays an important environmental role in the regional, and potentially the global energy, water and carbon balances. In many areas, however, miombo resources are threatened by clearing, grazing, illegal logging and uncontrolled fires (Campbell et al., 2007; Dewees et al., 2011).

* Corresponding author. Tel.: +351 214544687; fax: +351 214544689.
E-mail addresses: aribeiro@itqb.unl.pt, aribeiro@iict.pt (A.I. Ribeiro).

0305-1978
http://dx.doi.org/10.1016/j.bse.2012.12.020

Located in northern Mozambique, the Niassa National Reserve (NNR) represents one of the least disturbed areas of Africa's deciduous miombo woodlands. Nonetheless, this important conservation area is characterized by a gradient of fire frequency distribution, wherein the north-eastern side is affected by annual fires and the western by bi- to tri-annual fires. Fires, mostly anthropogenic, are strongly related with rainfall, vegetation, elephant and human density (Ribeiro, 2007; Ribeiro et al., 2009). In areas of higher fire frequency alterations on woodlands structure and composition are notable, with a decrease in woody parameters and a replacement of typical miombo species [*Julbernardia globiflora* (Benth.) Troupin and *Brachystegia* spp.] by sub-dominant species [*Combretum* spp., *Terminalia sericea* Burch. ex DC and *Diplorhynchus condylocarpon* (Muell. Arg.) Pichon] (Ribeiro et al., 2008). This may result in ecological disturbances in the future, whose impact is yet unpredictable. Under this scenario, an integrated fire management Program is being designed for NNR in order to sustainably manage the woodlands in this important area. Among others, the Program includes genetic diversity studies in priority tree species along the fire gradient. This will contribute to determine conservation needs for the selected species.

The use of molecular markers is an effective way to evaluate genetic variation within species because they are expedite and precise, and are not affected by the environment or biological processes. Polymerase Chain Reaction (PCR)-based markers, like Random Amplified Polymorphic DNA (RAPDs), Amplified Fragment Length Polymorphism (AFLP), Inter Simple Sequence Repeats (ISSRs), Simple Sequence Repeats (SSR) or Expressed Sequence Tags (EST)–SSR, are commonly used to assess genetic diversity in a wide range of plant species, including forest trees (Spooner et al., 2005; Ellis and Burke, 2007; Mondini et al., 2009; Abdul Kareem et al., 2012; Pillai et al., 2012; Yao et al., 2012). Of these, ISSR markers offer a powerful level of information due to their high effective multiplex ratio, comparable to AFLPs, coupled with the high expected heterozygosity levels delivered by microsatellite-based markers (Goulao and Oliveira, 2001). In addition, ISSRs are reproducible and technically easy to obtain.

In this paper we report on the usefulness of ISSR markers to assess genetic diversity in two typical miombo woodlands species, *Brachystegia boehmii* Taub. and *Burkea africana* Hook. f. for which information about the level of biodiversity has never been reported before. These species have a high commercial timber value and are used by rural dwellers in traditional medicine as well as for honey collection and fuel production, representing important ecological and socio-economic forest resources. Both have been described to be contrasting with respect to fire tolerance (Trapnell, 1959; Cauldwell and Ziegler, 2000), allowing an assessment of the possible impact of fires on the structure of the populations. Such studies will provide an important contribution to assist the definition of specific management practices for biodiversity conservation.

2. Materials and methods

2.1. Plant material and DNA extraction

Samples were collected in the Niassa National Reserve (NNR), located in northern Mozambique between the parallels 12°38′48.67″S and 11°27′05.83″S and the meridians 36°25′21.16″E and 38°30′23.74″E (Ribeiro, 2007). Young undamaged leaves were sampled from *B. africana* and from *B. boehmii* (15 and 14 individuals, respectively). Leaves were stored in silica gel rubin (Fluka) for about one week (the time of the trip from the field to the lab) and then frozen in liquid nitrogen and stored at −80 °C until DNA extraction.

Genomic DNA was extracted from 50 to 100 mg of ground leaves with the DNeasy Plant Mini Kit (Qiagen, Germany). DNA extraction was preceded by four washing steps with HEPES buffer (100 mM HEPES, 1% PVP, 2% β-Mercaptoethanol), by centrifuging for 1 min at the maximal speed in a bench mini-centrifuge, to remove contaminating polysaccharides. After RNase treatment (Fermentas, EU), the average yield and purity were assessed spectrophotometrically by OD_{230}, OD_{260} and OD_{280} readings (Lambda EZ201, Perkin Elmer, USA) and visually after 1% agarose electrophoresis. Each stock DNA was diluted to a working solution of 25 ng/μl.

2.2. ISSR amplification

Twenty seven primers (STAB Vida, Portugal) were initially screened using four randomly selected accessions (two from each sector) per species. Based on their amplification results, 8 primers that produced successful and solid amplification patterns were selected for the examination of the whole sample set (Table 1). PCRs were performed in 20 μl mixtures containing 50 ng of DNA, 1 μM of primer, 1 U *Taq* DNA polymerase (Fermentas, EU), 0.5 mM dNTP, and 2.5 mM $MgCl_2$, in 1× buffer (75 mM Tris–HCl pH 8.8 at 25 °C; 20 mM $(NH_4)_2SO_4$; 0.01% (v/v) Tween 20). Thermal cycling was performed with a BioRad iCycler using the following conditions: 1 cycle at 95 °C for 3 min followed by 35 cycles at 95 °C for 1.5 min, 42 °C for 1.5 min, 72 °C for 1.5 min, and a final extension at 72 °C for 5 min. PCR products were fractionated on 6% non-denaturing polyacrylamide gels (19:1 acrylamide:bis-acrylamide) containing 6.7 M urea, in 1× TBE buffer and visualized after silver staining, according to Bassam et al. (1991).

2.3. Phenetic analysis

ISSR data were transformed in a binary matrix consisting of "0"and "1" (absence and presence of bands, respectively), and analyzed for Clustering and Principal Coordinates Analysis (PCoA) using the software NTSYSpc version 2.20e (Rohlf, 2005). The unweight pair group method using arithmetic averages (UPGMA) and sequential agglomerative hierarchical nested

Table 1
Primer sequence, total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), average number of bands, average number of polymorphic bands and resolving power (Rp).

Primer	Sequence (5′–3′)	TNB	NPB	P%	Rp
Burkea africana					
ISSR-1	$(AG)_8$YT	11	9	81.8	13.20
ISSR-2	(AGC)4YT	14	14	100.0	15.07
ISSR-3	(CA)8R	9	9	100.0	9.07
ISSR-5	DBD $(CA)_7$	11	11	100.0	14.80
ISSR-6	VHV (GT)6	10	10	100.0	6.80
ISSR-7	HVH (TG)7	11	9	81.8	13.33
ISSR-8	HVH (CA)7	11	11	100.0	13.20
ISSR-10	(AC)8YT	13	13	100.0	6.80
TOTAL		90	86		
AVERAGE		11.25	10.75	95.6	11.53
Brachystegia boehmii					
ISSR-1	(AG)8YT	11	11	100.0	5.29
ISSR-5	DBD(CA)7	12	9	75.0	9.43
ISSR-7	HVH(TG)7	13	12	92.3	7.29
ISSR-10	(AC)8YT	7	7	100.0	4.57
ISSR-24	(GA)8YC	12	7	58.3	12.00
ISSR-25	(AG)8GYT	11	11	100.0	4.43
ISSR-26	(AG)8YC	15	15	100.0	9.14
ISSR-27	(AG)8YA	13	12	92.3	10.14
TOTAL		94	84		
AVERAGE		11.75	10.50	89.4	7.79

R = A/G, Y = C/T, K = T/G, B = T/C/G, D = A/T/G, V = A/C/G; H = A/T/C.

(SAHN) routines were performed based on the DICE's similarity coefficient (Nei and Li, 1979) for cluster analysis. Goodness of fit of the cluster analyses was determined by computing a cophenetic value matrix for the dendrograms and comparing with the original similarity matrix. The genetic similarity data matrix between accessions was also utilized to perform PCoA, using the DCENTER to transform the symmetric matrix to scalar product and EIGEN to extract eigenvectors modules of the NTSYSpc software. Polymorphic information content (PIC) and resolving power (Rp) were calculated as described by Botstein et al. (1980) and Prevost and Wilkinson (1999), respectively. The levels of within-population genetic diversity (H), estimates of total genetic diversity from both populations (Ht), mean diversity within populations (Hs), coefficient of gene differentiation (Gst), estimation of gene flow (Nm) and Shannon's gene diversity index were calculated using POPGENE32 (Yeh et al., 1997) using dominant and diploid parameters, on the basis of the Nei (1973) method and assuming the Hardy–Weinberg equilibrium. Genetic variation among populations and among genotypes within the populations was accessed through analysis of molecular variance (AMOVA) (Excoffier et al., 1992), at 0.05 significance level, using GenAlEx 6.41 (Peakall and Smouse, 2006).

3. Results

3.1. ISSR information content

Using 8 selected ISSR primers per species, 94 and 90 scorable fragments were obtained among the *B. boehmii* and *B. africana* genotypes. Even though the number of scorable ISSR markers did not differ too much between the two species a higher percentage of polymorphisms was observed in *B. africana*, i.e. 95.6% (86 bands) versus 89.4% (84 bands) in *B. boehmii*. The percentage of polymorphic loci in *B. africana* was 60.0% and 90.0% in the east and west sectors, respectively. Contrarily, in the case of *B. boehmii*, the percentage of polymorphic loci was lower in the western sector (48.9%) than in the eastern (68.1%).

Considering all individuals, regardless of the sampling side, the number of amplification products ranged from 9 (primer ISSR-3) to 14 (primer ISSR-2) in *B. africana* and from 7 (primer ISSR-10) to 15 (primer ISSR-26) in *B. boehmii*. All products scored with primers ISSR-2, -3, -5, -6, -8 and -10 (*B. africana*) and ISSR-1, -10, -25 and -26 (*B. boehmii*) were polymorphic. The information on the total number of bands, number and percentage of polymorphic bands, average number of bands, average number of polymorphic bands and resolving power obtained for each primer is given in Table 1.

3.2. Phenetic diversity

A dendrogram based on UPGMA analysis was generated using the Nei and Li's coefficient showing the clustering pattern between the individuals (Figs. 1 and 2). The similarity coefficient ranged from 0.4557 to 0.9159 (*B. africana*) and from 0.4375 to 0.8358 (*B. boehmii*). For both species four main clusters (I–IV) were formed, with eight, two, one and three individuals (*B. africana*) (Fig. 1A) and seven, two, three and two individuals (*B. boehmii*) (Fig. 2A). The cophenetic coefficient computed against the original data matrix was 0.93202 ($t = 4.5339$; $p = 1.0000$) for *B. africana* and 0.75478 ($t = 4.7265$; $p = 1.0000$) for *B. boehmii*, supporting the analysis. The results of the PCoA were comparable to the cluster analysis (Figs. 1B and 2B). The first three most informative PCo explained 50.87% of the total variation (25.45%, 13.53% and 11.89% for PCo1, PCo2 and PCo3,

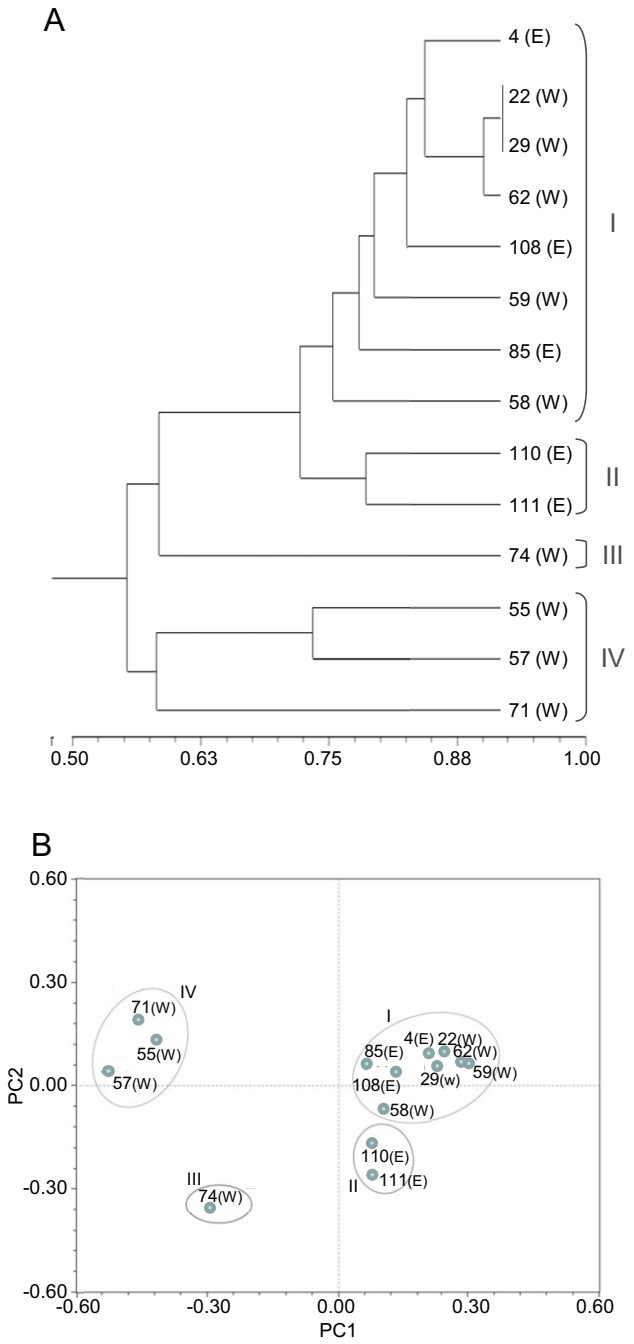

Fig. 1. Dendrogram based on UPGMA analysis generated using the Nei and Li's coefficient (A) and Principal Coordinate Analysis (PCoA) (B) representing phenetic relationships among the *B. africana* genotypes.

respectively) in *B. africana* and 49.86% of the total variation (20.48%, 17.24% and 12.14% for PCo1, PCo2 and PCo3, respectively) in *B. boehmii*.

In the case of *B. africana*, both UPGMA and PCo analyses showed a clear separation of individuals according to their origin in clusters II–IV. Cluster I grouped individuals from the east and west sectors. However, individuals #22, #29 and #66, all from the west sector of the reserve, presented more similarity, forming a sub-group within cluster I. Regarding *B. boehmii*, in general, the individuals clustered together according to their origin. The exceptions were individual #2, which grouped in cluster I together with individuals collected in the west region and cluster IV, which was composed by one individual from the east side (#79) and one individual from the west (#31).

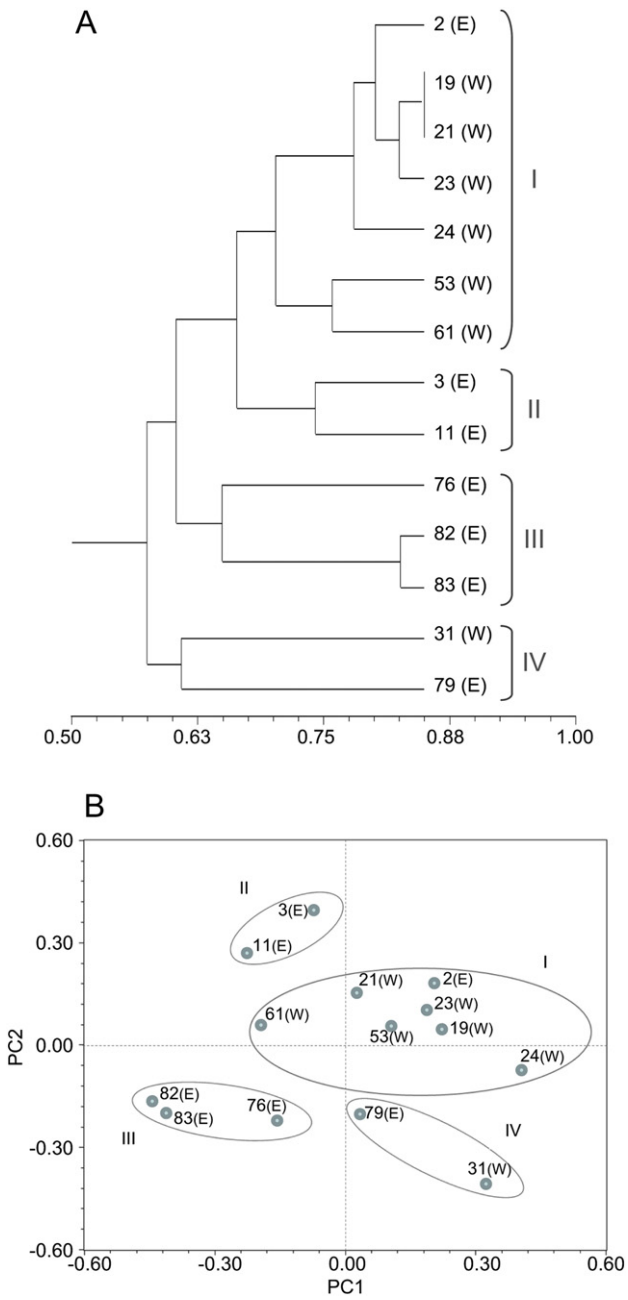

Fig. 2. Dendrogram based on UPGMA analysis generated using the Nei and Li's coefficient (A) and Principal Coordinate Analysis (PCoA) (B) representing phenetic relationships among the *B. boehmii* genotypes.

The population statistics generated by the ISSR markers (Table 2) includes data for the observed number of alleles, effective number of alleles, Nei's genetic diversity (expected heterozygosity), Shannon's information index and polymorphic information content. For *B. africana*, the average genetic heterozygosity was 0.2972, ranging from an average of 0.2977 in the west to 0.2184 in the east region, indicating more diversity in the western group. Contrarily, for *B. boehmii*, the average genetic heterozygosity was 0.1965, ranging from an average of 0.1482 in the west to 0.2059 in the east.

The Nei's (1978) unbiased genetic identity and distance of the west and east populations were respectively, 0.9374 and 0.0646 for *B. africana* and 0.9691 and 0.0314 for *B. boehmii*. The value for total genotype diversity among the east and west populations was found to be 0.1978 ± 0.0255 (*B. africana*) and 0.2905 ± 0.0254 (*B. boehmii*), while within-population diversity was 0.1771 ± 0.0209 (*B. africana*) and 0.2581 ± 0.0224 (*B. boehmii*). The mean coefficient of gene differentiation value for *B. africana* (0.1117) and *B. boehmii* (0.1049) indicated that 11.2% and 10.5%, respectively, of the genetic diversity resided

Table 2
Effective number of alleles (Ne), Nei's genetic diversity (He), Shannon's information index (I), overall genetic diversity (Ht), subpopulation genetic diversity (Hs), genetic differentiation among populations (Gst), estimate of gene flow from Gst (Nm) and polymorphic information content (PIC).

Locus	Ne	He (Total)	He (East)	He (West)	I	Ht	Hs	Gst	Nm	PIC
Burkea africana										
1	1.071	0.0663	0	0.1078	0.1494	0.0556	0.0539	0.0294	16.4853	0.0089
2	1.2331	0.1891	0.1591	0.2083	0.3374	0.1842	0.1837	0.0026	191.7017	0.0800
3	1.376	0.2733	0.4832	0	0.4451	0.3249	0.2416	0.2565	1.4495	1.7422
4	1.8609	0.4626	0.4832	0.4444	0.6553	0.4666	0.4638	0.006	82.6413	1.5022
5	1.376	0.2733	0.4832	0	0.4451	0.3249	0.2416	0.2565	1.4495	1.7422
6	1.1478	0.1288	0.1591	0.1078	0.2515	0.1339	0.1335	0.0033	148.9001	0.0356
7	1.376	0.2733	0.4832	0	0.4451	0.3249	0.2416	0.2565	1.4495	1.7422
8	1.6963	0.4105	0.1591	0.488	0.6008	0.3798	0.3236	0.1482	2.8741	0.4356
9	1.071	0.0663	0	0.1078	0.1494	0.0556	0.0539	0.0294	16.4853	0.0089
10	1	0	0	0	0	0	0	****	****	2.0000
11	1	0	0	0	0	0	0	****	****	2.0000
12	1.3296	0.2479	0.1591	0.2997	0.4139	0.234	0.2294	0.0198	24.695	0.1422
13	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
14	1.8747	0.4666	0.4142	0.488	0.6594	0.4595	0.4511	0.0183	26.794	0.7200
15	1.2331	0.1891	0.1591	0.2083	0.3374	0.1842	0.1837	0.0026	191.7017	0.0800
16	1.6506	0.3941	0.4142	0.3796	0.5832	0.3976	0.3969	0.0018	271.2964	0.4356
17	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
18	1.9068	0.4756	0.4832	0.3796	0.6685	0.4882	0.4314	0.1164	3.796	0.7200
19	1.9771	0.4942	0.4832	0.4984	0.6873	0.4928	0.4908	0.004	123.0383	1.2800
20	1.9231	0.48	0	0.4444	0.673	0.4444	0.2222	0.5	0.5	1.0756
21	1.8276	0.4528	0	0.488	0.6452	0.4107	0.244	0.4058	0.7321	1.2800
22	1.6826	0.4057	0	0.4984	0.5956	0.3603	0.2492	0.3084	1.1213	1.5022
23	1.978	0.4944	0	0.3796	0.6876	0.4676	0.1898	0.5941	0.3416	0.8889
24	1.9685	0.492	0.4832	0.4444	0.6851	0.4972	0.4638	0.0672	6.9452	0.8889
25	1.9068	0.4756	0.4832	0.3796	0.6685	0.4882	0.4314	0.1164	3.796	0.7200
26	1.7641	0.4331	0.4142	0.4444	0.6247	0.4301	0.4293	0.0019	262.5219	0.5689
27	1.0721	0.0673	0.1591	0	0.1512	0.0833	0.0795	0.0455	10.4772	0.0089
28	1.8716	0.4657	0.488	0.4444	0.6585	0.4702	0.4662	0.0085	58.4449	0.7200
29	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
30	1.8609	0.4626	0.4832	0.4444	0.6553	0.4666	0.4638	0.006	82.6413	1.5022
31	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
32	1.9992	0.4998	0.4832	0.488	0.693	0.4999	0.4856	0.0286	16.9817	1.0756
33	1.9771	0.4942	0.4832	0.4984	0.6873	0.4928	0.4908	0.004	123.0383	1.2800
34	1.9685	0.492	0.4832	0.4444	0.6851	0.4972	0.4638	0.0672	6.9452	0.8889
35	1.071	0.0663	0	0.1078	0.1494	0.0556	0.0539	0.0294	16.4853	0.0089
36	1.9626	0.4905	0.488	0.4444	0.6836	0.496	0.4662	0.06	7.8301	1.2800
37	1.9533	0.488	0.488	0.488	0.6811	0.488	0.488	0	2000	0.8889
38	1.5414	0.3512	0.4142	0.2997	0.5359	0.3629	0.3569	0.0165	29.8289	0.3200
39	1.8609	0.4626	0.4832	0.4444	0.6553	0.4666	0.4638	0.006	82.6413	1.5022
40	1.8609	0.4626	0.4832	0.4444	0.6553	0.4666	0.4638	0.006	82.6413	1.5022
41	1.2331	0.1891	0.1591	0.2083	0.3374	0.1842	0.1837	0.0026	191.7017	0.0800
42	1.8276	0.4528	0	0.488	0.6452	0.4107	0.244	0.4058	0.7321	1.2800
43	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
44	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
45	1.6826	0.4057	0	0.4984	0.5956	0.3603	0.2492	0.3084	1.1213	1.5022
46	1.071	0.0663	0	0.1078	0.1494	0.0556	0.0539	0.0294	16.4853	0.0089
47	1.1478	0.1288	0.1591	0.1078	0.2515	0.1339	0.1335	0.0033	148.9001	0.0356
48	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
49	1.9626	0.4905	0.488	0.4444	0.6836	0.496	0.4662	0.06	7.8301	1.2800
50	1.1478	0.1288	0.1591	0.1078	0.2515	0.1339	0.1335	0.0033	148.9001	0.0356
51	1.9992	0.4998	0.4832	0.488	0.693	0.4999	0.4856	0.0286	16.9817	1.0756
52	1.8276	0.4528	0	0.488	0.6452	0.4107	0.244	0.4058	0.7321	1.2800
53	1.3296	0.2479	0.1591	0.2997	0.4139	0.234	0.2294	0.0198	24.695	0.1422
54	1.2437	0.196	0	0.2997	0.3467	0.1667	0.1498	0.101	4.4495	0.0800
55	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
56	1.4279	0.2997	0.2997	0.2997	0.4767	0.2997	0.2997	0	2000	0.2222
57	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
58	1.2608	0.2069	0.4142	0	0.3612	0.25	0.2071	0.1716	2.4142	0.0800
59	1.9231	0.48	0	0.4444	0.673	0.4444	0.2222	0.5	0.5	1.0756
60	1	0	0	0	0	0	0	****	****	2.0000
61	1.9685	0.492	0.4832	0.4444	0.6851	0.4972	0.4638	0.0672	6.9452	0.8889
62	1.8609	0.4626	0.4832	0.4444	0.6553	0.4666	0.4638	0.006	82.6413	1.5022
63	1.6735	0.4025	0.488	0.2997	0.5922	0.4224	0.3938	0.0677	6.8865	0.4356
64	1.2331	0.1891	0.1591	0.2083	0.3374	0.1842	0.1837	0.0026	191.7017	0.0800
65	1	0	0	0	0	0	0	****	****	2.0000
66	1.346	0.2571	0.4142	0.1078	0.4253	0.2888	0.261	0.0962	4.6985	0.1422
67	1.346	0.2571	0.4142	0.1078	0.4253	0.2888	0.261	0.0962	4.6985	0.1422

(*continued on next page*)

Table 2 (*continued*)

Locus	Ne	He (Total)	He (East)	He (West)	I	Ht	Hs	Gst	Nm	PIC
68	1.5741	0.3647	0.488	0.2083	0.551	0.3945	0.3482	0.1176	3.7533	0.3200
69	1.8716	0.4657	0.488	0.4444	0.6585	0.4702	0.4662	0.0085	58.4449	0.7200
70	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
71	1.9771	0.4942	0.4832	0.4984	0.6873	0.4928	0.4908	0.004	123.0383	1.2800
72	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
73	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
74	1.4706	0.32	0	0.4444	0.5004	0.2778	0.2222	0.2	2	1.7422
75	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0356
76	1.3278	0.2469	0.2997	0.2083	0.4126	0.2561	0.254	0.0084	59.3408	0.1422
77	1.5414	0.3512	0.4142	0.2997	0.5359	0.3629	0.3569	0.0165	29.8289	0.3200
78	1.071	0.0663	0	0.1078	0.1494	0.0556	0.0539	0.0294	16.4853	0.0089
79	1.1478	0.1288	0.1591	0.1078	0.2515	0.1339	0.1335	0.0033	148.9001	0.0356
80	1.238	0.1922	0.2997	0.1078	0.3417	0.2117	0.2038	0.0377	12.7704	0.0800
81	1.7861	0.4401	0.2997	0.488	0.632	0.4224	0.3938	0.0677	6.8865	0.5689
82	1.6092	0.3786	0	0.488	0.5662	0.3333	0.244	0.2679	1.366	0.3200
83	1.071	0.0663	0	0.1078	0.1494	0.0556	0.0539	0.0294	16.4853	0.0088
84	1.9771	0.4942	0.4832	0.4984	0.6873	0.4928	0.4908	0.004	123.0383	1.28
85	1.1478	0.1288	0.1591	0.1078	0.2515	0.1339	0.1335	0.0033	148.9001	0.0356
86	1.2331	0.1891	0.1591	0.2083	0.3374	0.1842	0.1837	0.0026	191.7017	0.08
87	1.3296	0.2479	0.1591	0.2997	0.4139	0.234	0.2294	0.0198	24.695	0.1422
88	1.3296	0.2479	0.1591	0.2997	0.4139	0.234	0.2294	0.0198	24.695	0.1422
89	1.1516	0.1317	0	0.2083	0.2558	0.1111	0.1041	0.0627	7.4686	0.0355
90	1.2437	0.196	0	0.2997	0.3467	0.1667	0.1498	0.101	4.4495	0.08
Average	**1.4969**	**0.2972**	**0.2184**	**0.2977**	**0.4542**	**0.2905**	**0.2581**	**0.1117**	**3.9753**	**0.7332**
SD	**0.3365**	**0.1588**	**0.2106**	**0.1685**	**0.2026**	**0.0254**	**0.0224**			**0.6834**
Brachystegia boehmii										
1	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
2	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
3	1.694	0.4097	0.4649	0	0.5999	0.4325	0.2325	0.4625	0.5811	0.8265
4	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
5	1.2254	0.1839	0.3492	0	0.3304	0.2	0.1746	0.127	3.4365	0.0408
6	1.3856	0.2783	0.4649	0	0.4512	0.3	0.2325	0.2251	1.7208	0.0918
7	1.9992	0.4998	0.4649	0.4832	0.6929	0.4992	0.474	0.0504	9.43	0.6530
8	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
9	1.2254	0.1839	0.3492	0	0.3304	0.2	0.1746	0.127	3.4365	0.0408
10	1.1006	0.0914	0.1889	0	0.1925	0.1	0.0944	0.0557	8.4721	0.0102
11	1.9007	0.4739	0	0.4142	0.6668	0.4571	0.2071	0.5469	0.4142	0.6530
12	1.5697	0.3629	0.4142	0.2997	0.549	0.3629	0.3569	0.0165	29.8289	0.2551
13	1	0	0	0	0	0	0	****	****	1.4693
14	1.9737	0.4933	0.4142	0.4832	0.6865	0.4933	0.4487	0.0905	5.0236	0.6530
15	1	0	0	0	0	0	0	****	****	1.4693
16	1.2	0.1667	0.2997	0	0.3066	0.1667	0.1498	0.101	4.4495	0.0408
17	1	0	0	0	0	0	0	****	****	1.4693
18	1.5697	0.3629	0.4142	0.2997	0.549	0.3629	0.3569	0.0165	29.8289	0.2551
19	1.2	0.1667	0	0.2997	0.3066	0.1667	0.1498	0.101	4.4495	0.0408
20	1.2	0.1667	0.2997	0	0.3066	0.1667	0.1498	0.101	4.4495	0.0408
21	1.3333	0.25	0	0.4142	0.4165	0.25	0.2071	0.1716	2.4142	0.0918
22	1.0909	0.0833	0.1591	0	0.1791	0.0833	0.0795	0.0455	10.4772	0.0102
23	1.1892	0.1591	0.1591	0.1591	0.2958	0.1591	0.1591	0	2000	0.0408
24	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
25	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
26	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
27	1.4	0.2857	0.4522	0	0.4601	0.2857	0.2261	0.2087	1.8956	0.1632
28	1.1667	0.1429	0.2617	0	0.2724	0.1429	0.1309	0.0839	5.458	0.0408
29	1.4959	0.3315	0.4522	0.1374	0.5136	0.3315	0.2948	0.1109	4.0086	0.2551
30	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
31	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
32	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
33	1.0769	0.0714	0.1374	0	0.1586	0.0714	0.0687	0.0385	12.4807	0.0102
34	1.4959	0.3315	0.4522	0.1374	0.5136	0.3315	0.2948	0.1109	4.0086	0.2551
35	1	0	0	0	0	0	0	****	****	2
36	1	0	0	0	0	0	0	****	****	2
37	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
38	1.173	0.1475	0.1591	0.1374	0.2792	0.1483	0.1482	0.0006	883.9095	0.0408
39	1.4063	0.2889	0.4142	0.1374	0.4639	0.2997	0.2758	0.0798	5.7653	0.1632
40	1.9108	0.4766	0.4832	0.4702	0.6696	0.4771	0.4767	0.001	519.7732	1.2346
41	1.9928	0.4982	0.488	0.4702	0.6913	0.499	0.4791	0.0398	12.052	1.0204
42	1.173	0.1475	0.1591	0.1374	0.2792	0.1483	0.1482	0.0006	883.9095	0.0408
43	1.173	0.1475	0.1591	0.1374	0.2792	0.1483	0.1482	0.0006	883.9095	0.0408
44	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102

Table 2 (*continued*)

Locus	Ne	He (Total)	He (East)	He (West)	I	Ht	Hs	Gst	Nm	PIC
45	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
46	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
47	1.1835	0.155	0.2997	0	0.2901	0.1667	0.1498	0.101	4.4495	0.0408
48	1.1835	0.155	0.2997	0	0.2901	0.1667	0.1498	0.101	4.4495	0.0408
49	1	0	0	0	0	0	0	****	****	2
50	1.3882	0.2796	0.2997	0.2617	0.4528	0.2811	0.2807	0.0015	341.7891	0.1632
51	1.785	0.4398	0.488	0.369	0.6317	0.4445	0.4285	0.0359	13.4372	0.5
52	1	0	0	0	0	0	0	****	****	1.7244
53	1	0	0	0	0	0	0	****	****	1.7244
54	1	0	0	0	0	0	0	****	****	1.7244
55	1	0	0	0	0	0	0	****	****	1.7244
56	1	0	0	0	0	0	0	****	****	0
57	1	0	0	0	0	0	0	****	****	0
58	1.1835	0.155	0.2997	0	0.2901	0.1667	0.1498	0.101	4.4495	0.0408
59	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
60	1.173	0.1475	0.1591	0.1374	0.2792	0.1483	0.1482	0.0006	883.9095	0.0408
61	1.1835	0.155	0.2997	0	0.2901	0.1667	0.1498	0.101	4.4495	0.0408
62	1.2958	0.2283	0	0.369	0.3891	0.2143	0.1845	0.139	3.0972	0.0918
63	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
64	1.1835	0.155	0.2997	0	0.2901	0.1667	0.1498	0.101	4.4495	0.0408
65	1.4797	0.3242	0	0.4702	0.5052	0.3065	0.2351	0.233	1.6458	1.4693
66	1.6523	0.3948	0.2997	0.4522	0.5839	0.389	0.3759	0.0337	14.3516	0.3673
67	1.2254	0.1839	0.3492	0	0.3304	0.2	0.1746	0.127	3.4365	0.0408
68	1.2254	0.1839	0.3492	0	0.3304	0.2	0.1746	0.127	3.4365	0.0408
69	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
70	1.7858	0.44	0.4649	0.4142	0.6319	0.4423	0.4396	0.0063	78.8763	0.3673
71	1.5295	0.3462	0	0.4832	0.5303	0.3249	0.2416	0.2565	1.4495	1.0204
72	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
73	1.9974	0.4993	0.4944	0.488	0.6925	0.4997	0.4912	0.0169	29.0059	0.6530
74	1.9992	0.4998	0.4649	0.4832	0.6929	0.4992	0.474	0.0504	9.43	0.6530
75	1.3374	0.2523	0.1889	0.2997	0.4194	0.2473	0.2443	0.0123	40.219	0.0918
76	1.1006	0.0914	0.1889	0	0.1925	0.1	0.0944	0.0557	8.4721	0.0102
77	1.9007	0.4739	0	0.4142	0.6668	0.4571	0.2071	0.5469	0.4142	0.6530
78	1.2197	0.1801	0	0.2997	0.3253	0.1667	0.1498	0.101	4.4495	0.0408
79	1.4791	0.3239	0.4944	0	0.5049	0.3472	0.2472	0.288	1.2361	1.0204
80	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
81	1.0995	0.0905	0	0.1591	0.1912	0.0833	0.0795	0.0455	10.4772	0.0102
82	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
83	1.5188	0.3416	0.4142	0.2617	0.5251	0.3475	0.338	0.0274	17.7349	0.2551
84	1.9767	0.4941	0.488	0.4976	0.6872	0.4937	0.4928	0.0019	268.6839	0.8265
85	1.3882	0.2796	0.2997	0.2617	0.4528	0.2811	0.2807	0.0015	341.7891	0.1632
86	1.1835	0.155	0.2997	0	0.2901	0.1667	0.1498	0.101	4.4495	0.0408
87	1.0831	0.0767	0	0.1374	0.1678	0.0714	0.0687	0.0385	12.4807	0.0102
88	1.0836	0.0772	0.1591	0	0.1686	0.0833	0.0795	0.0455	10.4772	0.0102
89	1.9867	0.4966	0.4832	0.4522	0.6898	0.498	0.4677	0.061	7.7025	0.8265
90	1.6718	0.4018	0.488	0.2617	0.5915	0.4107	0.3749	0.0873	5.2271	0.3673
91	1	0	0	0	0	0	0	****	****	1.7244
92	1.9009	0.4739	0.4142	0.4976	0.6668	0.4708	0.4559	0.0316	15.3067	0.6530
93	1.9929	0.4982	0.4142	0.4702	0.6914	0.4964	0.4422	0.1091	4.0819	0.8265
94	1.2765	0.2166	0.1591	0.2617	0.374	0.2127	0.2104	0.0108	45.8846	0.0918
Average	**1.3055**	**0.1965**	**0.2059**	**0.1482**	**0.3193**	**0.1978**	**0.1771**	**0.1049**	**4.2648**	**0.4016**
SD	**0.3146**	**0.1601**	**0.179**	**0.1803**	**0.2168**	**0.0255**	**0.0209**			**0.5854**

between populations. The estimated inter-population gene flow was 3.9753 (*B. africana*) and 4.2648 (*B. boehmii*), which shows extensive exchange among populations. Likewise, AMOVA analysis revealed that almost all the molecular variance (97% for *B. africana* and 93% for *B. boehmii*) was found to be within populations and only 3% and 7%, respectively, was among populations (Table 3), indicating that there is more variation within the populations.

4. Discussion

In this study we have used ISSR markers in order to assess the genetic diversity of two typical miombo species, *B. africana* and *B. boehmii*. According to our data, ISSR seem to be a promising molecular marker for biodiversity studies in both species, disclosing high levels of polymorphisms (95.6% and 89.4% for *B. africana* and *B. boehmii*, respectively) and displaying a convenient resolving power (11.53 and 7.79 for *B. africana* and *B. boehmii*, respectively). ISSRs are amongst the most reliable molecular markers for genetic diversity studies, being highly reproducible and generating a substantial number of polymorphisms (Bornet and Branchard, 2001; Myśków et al., 2010). This marker system is particularly important for species like *B.*

Table 3
Analysis of molecular variance (AMOVA) from a comparison of the ISSR profiles from *B. africana* and *B. boehmii* individuals collected from two populations (east and west). *P* is the probability of obtaining a larger variance value.

Species	Source	Df	SS	MS	Variance	% total variation	F_{st}	*P*
B. africana	Among populations	1	17.131	17.131	0.456	3	0.170	0.032
B. africana	Within populations	13	180.056	13.850	13.850	97	–	–
B. boehmii	Among populations	1	18.539	18.539	0.918	7	0.071	0.040
B. boehmii	Within populations	12	145.314	12.110	12.110	93	–	–

africana and *B. boehmii*, from which no genome information is available and no similar studies have been yet described. In fact, opposite to SSR and EST–SSRs, the development of ISSR markers does not require prior knowledge of the genome sequence or information on species specific sequences, but still produces comparable high heterozygosity levels. On the other hand, the reproducibility and costs of ISSR markers are quite well balanced as compared to RAPD (which are described to have reproducibility problems) and AFLP (which are cost- and labor-intensive) (Wu et al., 1994; Zietkiewicz et al., 1994; Myśków et al., 2010).

The distribution of the sampled individuals by clusters, supported by PCo analysis, suggests a divergent adaptation response to distinct environments. Estimation of gene diversity showed that the available genetic variability across the fire gradient is different for the two species. In fact, while in the case of *B. africana* the western population showed higher variability than that from the east, the opposite was observed for *B. boehmii*. The contrasting percentage of ISSR polymorphic bands in the east region when compared to the west sector (60.0% versus 90.0% for *B. africana* and, oppositely, 68.1% versus 48.9% for *B. boehmii*) supports this observation. This opposite trends may be related to fire tolerance and adaptation. *B. africana* is a typical fire-tolerant species while *B. boehmii* is sensitive to fires, particularly at young stages (Trapnell, 1959; Cauldwell and Ziegler, 2000). The results are supported by the ecological analysis, i.e.: i) comparable Importance Value Index (IVI) for *B. africana* in both sectors, indicating that the abundance of this species within the ecosystem was minimally affected by fires; and ii) lower IVI values in eastern versus western sectors for *B. boehmii* indicating that, under higher fire frequency, the relative abundance of this species within the ecosystem is compromised, with an increased dominance of fire-tolerant species (Natasha Ribeiro, Gisela Cuambe, Aires Banze and Ana Ribeiro, unpublished data). Thus, in areas of high fire frequency the putative negative impacts are expected be more prominent in *B. boehmii*. Even so, high fire frequency seems to have a negative impact in the overall genetic pool of *B. africana* leading to a decrease in the degree of genetic diversity. Regarding the fire-sensitive *B. boehmii*, the higher levels of variation in the eastern population might be the result of an increase in its genetic pool, which, during evolution, allowed the species survival facing a higher incidence of fires. This could be explained by a pyrodiversity-like effect (Parr and Andersen, 2006). Moreover, it may be explained by a higher proportion of seed-derived propagation a way to cope with fires that affect the survival of young individuals, disturbing vegetative propagation.

The total genetic diversity was slightly higher among populations which may be explained by genetic drift or due to small sample size. It should be noted that the sampling used in this study is not representative of the entire area, so the results reported here are of paramount importance to assist the research team in improving sample collection, namely by validation of new techniques, especially for miombo woodlands, providing an insight into its applicability and suggesting future directions of research.

Nevertheless, the relatively low proportion of total variation associated with inter-population differentiation (mean coefficients of gene differentiation = 0.1117 and 0.1049 for *B. africana* and *B. boehmii*, respectively), indicates that a substantial proportion of genetic diversity (88.2% and 89.5%) lies among individual accessions. The estimated gene flow for the two populations (inter-population gene flow = 3.9753 and 4.2648 for *B. africana* and *B. boehmii*, respectively) was high and possibly account for the similarity within and among these populations in both species. These results might reflect a well preserved genetic pool in NNR, agreeing with the fact that the reserve is one of least disturbed areas of miombo.

In conclusion, genetic diversity analysis suggests that, i) there is a good genetic potential for *B. africana* and *B. boehmii* in both eastern and western NNR; and ii) the current fire frequency is affecting differently typical miombo species. As part of the Biodiversity Conservation Program of NNR, we are currently extending our studies to other sites of NNR and to other priority species in order to better understand the ecosystem dynamics and its conservation status within a fire context.

Acknowledgments

The authors are grateful to Dr. Anabela Rodrigues, manager of the *Sociedade de Gestão e Desenvolvimento da Reserva Nacional do Niassa* (SGDRN) for helping in prioritizing the research lines; *Fundo Nacional de Investigação*, Ministry of Science and Technology, Mozambique (Project 25A); Bioversity International/CGIAR through the Portuguese contribution (Science and Technology Foundation, Ministry of Science, Technology and Higher Education); Isabel Moura (Tropical Research Institute – IICT), Cândida Zitha and Esménia Amaral (Faculty of Agronomy and Forest Engineering, Eduardo Mondlane University) for field assistance; Isabel Palos (IICT) for administrative assistance, and Carmen Gracia-Fernandez (Bioversity International) for the helpful discussions.

References

Abdul Kareem, V.K., Rajasekharan, P.E., Ravish, B.S., Mini, S., Sane, A., Vasantha Kumar, T., 2012. Analysis of genetic diversity in *Acorus calamus* populations in South and North East India using ISSR markers. Biochem. Syst. Ecol. 40, 156–161.

Bassam, B.J., Caetano-Anollés, G., Gresshoff, P.M., 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal. Biochem. 196, 80–83.

Bornet, B., Branchard, M., 2001. Nonanchored inter-simple sequence repeat (ISSR) markers: reproducible and specific tools for genome fingerprinting. Plant Mol. Biol. Rep. 19, 209–221.

Botstein, D., White, R.L., Skolnick, M., Davis, R.W., 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am. J. Hum. Genet. 32, 314–331.

Campbell, B., Angelsen, A., Cunningham, A., Katerere, Y., Sitoe, A., Wunder, S., 2007. Miombo Woodlands: Opportunities and Barriers to Sustainable Forest Management. Center for International Forestry Research, Bogor.

Cauldwell, A.E., Ziegler, U., 2000. A reassessment of the fire-tolerance of some miombo woody species in the Central Province, Zambia. Afr. J. Ecol. 38, 138–146.

Dewees, P., Campbell, B., Katerere, Y., Sitoe, A., Cunningham, A.B., Angelsen, A., Wunder, S., 2011. Managing the Miombo Woodlands of Southern Africa: Policies, Incentives, and Options for the Rural Poor. Program on Forests (PROFOR), Washington, DC.

Ellis, J.R., Burke, J.M., 2007. EST-SSRs as a resource for population genetic analyses. Heredity 99, 125–132.

Excoffier, L., Smouse, P.E., Quattro, J.M., 1992. Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. Genetics 131, 479–491.

Goulao, L., Oliveira, C.M., 2001. Molecular characterisation of cultivars of apple (*Malus* × *domestica* Borkh.) using microsatellite (SSR and ISSR) markers. Euphytica 122, 81–89.

Mondini, L., Noorani, A., Pagnotta, M.A., 2009. Assessing plant genetic diversity by molecular tools. Diversity 1, 19–35.

Myśków, B., Milczarski, P., Masojć, P., 2010. Comparison of RAPD, ISSR and SSR markers in assessing genetic diversity among rye (*Secale cereal* L.) inbred lines. Plant Breed. Seed Sci. 62, 101–115.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proc. Natl. Acad. Sci. U.S.A. 70, 3321–3323.

Nei, M., 1978. Estimation of average heterozygosity and genetic distance from a small number of individuals. Genetics 89, 583–590.

Nei, M., Li, W.H., 1979. Mathematical model for studying genetic variation in terms of restriction endonucleases. Proc. Natl. Acad. Sci. U.S.A. 76, 5269–5273.

Parr, C.L., Andersen, A.N., 2006. Patch mosaic burning for biodiversity conservation: a critique of the pyrodiversity paradigm. Conserv. Biol. 20, 610–619.

Peakall, R., Smouse, P.E., 2006. GENALEX 6: genetic analysis in Excel. Population genetic software for teaching and research. Mol. Ecol. Notes 6, 288–295.

Pillai, P.P., Sajan, J.S., Menon, K.M., Jayakumar, K.S.P., Subramoniam, A., 2012. SSR analysis reveals high intraspecific variation in *Rauvolfia serpentina* L. – a high value medicinal plant. Biochem. Syst. Ecol. 40, 192–197.

Prevost, A., Wilkinson, M.J., 1999. A new system of comparing PCR primers applied to ISSR fingerprinting of potato cultivars. Theor. Appl. Genet. 98, 107–112.

Ribeiro, N.S., 2007. Interactions between fires and elephants in relation to vegetation composition and structure of miombo woodlands in northern Mozambique. PhD dissertation, Environmental Sciences. University of Virginia, USA.

Ribeiro, N.S., Shugart, H.H., Washington-Allen, R.A., 2008. The effects of fire and elephants on species composition and structure of the Niassa Reserve, northern Mozambique. J. For. Ecol. Manage. 255, 1626–1636.

Ribeiro, N.S., Okin, G.S., Shugart, H.H., Swap, R.J., 2009. The influence of rainfall, vegetation, elephants and people on fire frequency of miombo woodlands, northern Mozambique. In: Proceedings of the International Geoscience and Remote Sensing Symposium, Cape Town.

Rohlf, F.J., 2005. NTSYS-pc: Numerical Taxonomy and Multivariate Analysis System, Version 2.2. Exeter Software, Setauket, New York.

Spooner, D., Treuren, R., Vicente, M.C., 2005. Molecular markers for genebank management. IPGRI Tech. Bull. 10

Trapnell, C.G., 1959. Ecological results of woodland burning experiments in northern Rhodesia. J. Ecol. 47, 129–168.

Wu, K., Jones, R., Dannaeberger, L., Scolnik, P.A., 1994. Detection of microsatellite polymorphisms without cloning. Nucleic Acids Res. 22, 3257–3258.

Yao, Q.L., Chen, F.B., Fang, P., Zhou, G.F., Fan, Y.H., Zhang, Z.R., 2012. Genetic diversity of Chinese vegetable mustard (*Brassica juncea* Coss) landraces based on SSR data. Biochem. Syst. Ecol. 45, 41–48.

Yeh, F.C., Yang, R.C., Boyle, T.B.J., Ye, Z.H., Mao, J.X., 1997. POPGENE, the User-friendly Shareware for Population Genetic Analysis. Molecular Biology and Biotechnology Centre, University of Alberta, USA.

Zietkiewicz, E., Rafalski, A., Labuda, D., 1994. Genome fingerprinting by simple sequence repeat (SSR) – anchored polymerase chain reaction amplification. Genomics 20, 176–183.